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Longitudinal assessment of DNA methylation changes during HPVE6E7-induced immortalization of primary keratinocytes

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Running title: Sequential DNA methylation during immortalization by seven HPV types

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Abbreviations and Acronyms

CIN	Cervical intraepithelial neoplasia
HFK	Human foreskin keratinocytes
hrHPV	High-risk human papillomavirus
LZRS	Empty vector
MIP	Methylation independent PCR
(q)MSP	(quantitative) methylation specific PCR
SCC	Squamous cell carcinoma
SFM	Serum free medium

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Abstract

High-risk human papillomavirus (hrHPV)-induced immortalization and malignant transformation is accompanied by DNA methylation of host genes. To determine when methylation is established during cell immortalization and whether it is hrHPV-type dependent, DNA methylation was studied in a large panel of HPVE6E7-immortalized keratinocyte cell lines. These cell lines displayed different growth behaviors, i.e. continuous growth versus crisis period prior to immortalization, reflecting differential immortalization capacities of the seven HPV-types (16/18/31/33/45/66/70) studied. In this study, cells were monitored for hypermethylation of 14 host genes (APC, CADM1, CYGB, FAM19A4, hTERT, miR124-1, miR124-2, miR124-3, MAL, PHACTR3, PRDM14, RASSF1A, ROBO3, and SFRP2) at 4 different stages during immortalization.

A significant increase in overall methylation levels was seen with progression through each stage of immortalization. At stage 1 (pre-immortalization) a significant increase in methylation of hTERT and miR124-2 and PRDM14 was already apparent, which continued over time. Methylation of ROBO3 was significantly increased at stage 2 (early immortal), followed by CYGB (stage 3) and FAM19A4, MAL, PHACTR3 and SFRP2 (stage 4). Methylation patterns were mostly growth behavior independent. Yet, hTERT methylation levels were significantly increased in cells that just escaped from crisis. Bisulfite sequencing of hTERT confirmed increased methylation in immortal cells compared to controls, with the transcription core and known repressor sites remaining largely unmethylated.

In conclusion, HPV-induced immortalization is associated with a sequential and progressive increase in promoter methylation of a subset of genes, which is mostly independent of the viral immortalization capacity.

Introduction

Infection with high-risk human papillomavirus (hrHPV) is the major cause of cervical cancer, as well as a subset of other anogenital cancers and head and neck cancers.^{1, 2} According to their prevalence in cervical cancer, twelve HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) are classified as high-risk and eight (HPV26, 53, 66, 67, 68, 70, 73 and 82) as probable or possible high-risk.¹

Cervical cancer develops via morphologically recognizable precancerous lesions, also known as cervical intraepithelial neoplasia (CIN). Cervical carcinomas and their closest precursor lesions, i.e. high-grade or transforming CINs, are characterized by elevated expression of the viral oncogenes E6 and E7 in proliferating cells.³ E6 and E7 are known to dysregulate apoptosis, cell cycle control and the replicative lifespan by interfering amongst others with activities of p53, pRB and hTERT.^{4, 5} This is further accompanied by the induction of genomic instability and epigenetic changes.⁴ Epigenetic alterations include both histone modifications and DNA methylation, which control the chromatin status and affect gene transcription.⁶

We and others have previously shown that various established and probable/possible hrHPV types display differential properties in terms of immortalization of primary human foreskin keratinocytes (HFK).⁷⁻⁹ In our studies, the E6/E7 genes of HPV16, 18, 31 and 33 consistently triggered a continuous growth without apparent growth arrest (crisis). Transduction of HFK by E6/E7 of HPV45, 66 and 70, on the other hand, resulted initially in an extended lifespan, but only after a long period of growth reduction or crisis some immortal clones emerged. In case of HPV45 only HFKs of one out of three donors ultimately became immortal. In all cultures, immortalization was characterized by upregulated expression of hTERT, the catalytic subunit of telomerase, and by activation of telomerase. The differential growth behaviors suggest that immortalization induced by HPV16, 18, 31 and 33 either requires less additive epigenetic or genetic events, or that the timing of these events is different, i.e. before normally a crisis period would become manifest. Candidate epigenetic events associated with HPV-induced cervical carcinogenesis include promoter methylation of various host cell genes, often resulting in silencing of the respective genes. Frequently methylated genes in cervical cancer include APC, CADM1, CYGB, FAM19A4, hTERT, miR124-1, miR124-2, miR124-3, MAL, PHACTR3, PRDM14, RASSF1A, ROBO3, and SFRP2 (for reviews see refs. 3, 10-12). A subset of the above mentioned genes (such as APC, CADM1, FAM19A4, hTERT, MAL, miR124-1, miR124-2, miR124-3, PHACTR3 and PRDM14) have previously been tested in HPV16- and 18-immortalized HFK cell lines to determine the onset and order of DNA methylation alterations during HPV16 and 18-induced transformation.¹³⁻¹⁸ A progressive increase in methylation levels with passaging of these cell lines was seen and methylation levels were generally higher in cervical cancer cell lines than in *in vitro* HPV16- or 18-immortalized HFKs. Furthermore, for CADM1, hTERT, MAL, miR124, PRDM14 and SFRP2 a functional role of methylation-mediated gene silencing in HPV-induced transformation could be demonstrated.¹⁵⁻²⁰ Whereas promoter hypermethylation of most of the above mentioned genes has been linked to gene silencing, hypermethylation of hTERT has been found to positively correlate to gene expression and activation.^{18, 21-24} This phenomenon may be attributed to methylation-mediated inhibition of the transcriptional repressor CTCF, which can bind to the hTERT gene in the first and second exon.²⁵ Since upregulated hTERT mRNA expression is critical to telomerase activation and HPV-induced immortalization,²⁶ hTERT methylation may represent an important regulatory mechanism of HPV-induced immortalization.

It is currently unknown to what extent the changes in methylation are related to the different stages of transformation induced by HPV types other than HPV16 and 18, and whether altered DNA methylation during immortalization is related to the oncogenic capacity of the different HPV types.

Since cells from various passages of HPV16-, 18-, 31-, 33-, 45-, 66- and 70-transduced keratinocytes have been harvested and stored, this offers unique possibilities to relate gene promoter hypermethylation to the immortalization capacities of these HPV types in a longitudinal manner. Here, we analyzed the methylation status of 14 host cell genes, i.e. APC, CADM1, CYGB, FAM19A4, hTERT, miR124-1, miR124-2, miR124-3, MAL, PHACTR3, PRDM14, RASSF1A, ROBO3, and SFRP2, at various passages pre- and post-immortalization. This included a more in depth methylation analysis of individual CpGs within the hTERT regulatory sequences.

Results

Promoter methylation increases during HPV-induced immortalization

Previously, we have shown that E6/E7 of HPV16, 18, 31, 33, 45, 66 and 70 have differential capacities to immortalize primary HFKs.⁸ HPV16-, 18-, 31- and 33-transduced HFKs showed a continuous growth, whereas immortalization of HFKs by HPV45, 66 and 70 was preceded by a long period of crisis. Accordingly, the latter types are considered less oncogenic in terms of *in vitro* immortalization.

To determine whether methylation of particular genes during the immortalization process is related to the observed variations in growth behavior, methylation was analyzed at four different stages of two or three HPV-transduced HFK donors (donor I-III) per HPV type. These involved the following stages: (1) passage 14-20, pre-immortal cells in their extended life span; (2) passage 25-30, early passage immortal, telomerase positive cells; (3) passage 40- 46, intermediate passage immortal, telomerase positive cells and (4) passage 73-80, late passage immortal, telomerase positive cells. Cultures were considered immortal in case of growth beyond a state where HFKs transduced by E6/E7 of HPV45, 66 and 70 were in crisis. All immortal cultures were characterized by prolonged growth, elevated hTERT mRNA expression and telomerase activity.⁸ For stage 1, 17 cell cultures were analyzed, including those of HPV45 transduced HFKs of donor II and III, which did not reach stage 2. For stage 2 to 4, 15 cell lines were examined. Early passages of untransduced HFKs (n=6), HFKs transduced with empty vector (LZRS; n=3) and HFK of donor I transduced with low-risk HPV11, that did not become immortal, were used as negative controls.⁸ The cancer cell lines, SiHa and A549 served as positive controls.^{13, 15-18, 27, 28}

Promoter methylation of 14 genes that are frequently methylated in (cervical) cancer,^{13, 15-18, 27} i.e. APC, CADM1, CYGB, FAM19A4, hTERT, miR124-1, miR124-2, miR124-3, MAL, PHACTR3, PRDM14, RASSF1A, ROBO3, and SFRP2, was determined by qMSP. For hTERT, two qMSPs, referred to as hTERT M1 and hTERT M2 that target the promoter region and first intron/second exon, respectively, were tested, given their putative association with methylation-mediated hTERT activation.^{18, 21-25}

The promoter regions of APC, miR124-1, miR124-3 and RASSF1A did not show any hypermethylation in the HPV-immortalized cell lines, whereas methylation levels were high in (cervical) cancer cell lines. For the remaining ten genes, CADM1, CYGB, FAM19A4, hTERT, miR124-2, MAL, PHACTR3, PRDM14, ROBO3, and SFRP2, increased promoter methylation was detected in the HPV-immortalized cells at least at one stage of transformation.

When comparing the averaged methylation level of all 14 genes a significant increase was seen with progression through each stage of transformation ($p < 0.01$ at each stage) (Figure 1A). Moreover, methylation levels at stage 1 were significantly increased compared to controls ($p < 0.01$) and the highest methylation levels were detected in cancer cells (Figure 1A).

The methylation levels per individual gene at stage 1 to 4 are shown in Figure 1B-L. At stage 1 a significant increase in methylation of hTERT M1 and M2, miR124-2 and PRDM14 compared to controls was already apparent (Figure 1E, F, H and J, respectively). Moreover, methylation of all three genes showed a progressive increase over time. Methylation of ROBO3 was significantly increased at stage 2 compared to stage 1 (Figure 1K) and methylation of CYGB was significantly increased at stage 3 compared to stage 1 (Figure 1C). FAM19A4, MAL, PHACTR3 and SFRP2 was significantly increased at stage 4 (Figure 1D, G, and I, respectively). For CADM1 a slight increase in methylation was seen with passaging, which did not reach significance in this relatively small sample set.

Progressive increase in levels of DNA methylation is mostly growth behavior independent

In order to determine whether the progressive increase in DNA methylation levels is related to the growth behavior of the cells, the average methylation level of all 14 genes at the individual stages were compared between cell lines that grew continuously (i.e. HPV16-, 18-, 31- and 33-immortalized cells) and cell lines that encountered a crisis period prior to immortalization (i.e. HPV45-, 66- and 70-immortalized cells). As shown in Figure 2A a borderline significant difference between both groups is seen at stage 2 ($p = 0.059$), which is just after immortalization. No clear differences were seen at stage 1, 3 and 4. Further comparison of individual gene methylation levels at stage 2 between cells with and without growth crisis, revealed that methylation of hTERT at the M2 region and methylation of PHACTR3 were both significantly increased in cells that underwent a crisis compared to those that grew continuously (Figure 2B,C).

Increased hTERT methylation in HPV-immortalized cell lines

As described above, methylation of hTERT represented one of the earliest methylation events in the majority of HPV-transduced HFKs and methylation at the M2 region was significantly increased in cells that just escaped crisis (i.e. at stage 2 in HPV45-, 66- and 70- immortalized cells) compared to cell that grew continuously (i.e. HPV16-, 18-, 31- and 33-immortalized cells). This difference leveled out at stage 3 and 4. The M2 region was included as it is located next to a CTCF binding site implicated in negative hTERT transcription regulation. Methylation of CTCF binding sequences has been shown to inhibit CTCF binding, thereby contributing to hTERT upregulation and telomerase activation.^{21, 22, 25} To determine in more detail which transcription factor binding sites in hTERT are targeted by DNA methylation in the HPV-immortalized cells, cells (stage 3) were subjected to bisulfite sequencing on 4 overlapping regions of the hTERT promoter and gene: -476 to -185 bp (region S1), -209 to +96 bp (region S2), +90 to +338 bp (region S3) and +319 to +600 bp (region S4) (top Figure 3). The methylation patterns in hTERT-positive HPV-immortalized cells were compared to HFKs and HPV11-transduced HFKs which we previously showed to be hTERT mRNA negative,⁸ and hTERT mRNA positive SiHa cervical cancer cells, in which the hTERT promoter is known to be highly methylated.¹⁸

As shown in Figure 3 and Table 1, an increase in methylated CpGs in hrHPV-immortalized cells compared to controls is particularly evident for region S1. Methylation in this region ranged from 7% to 22% in HPV-immortalized cells compared to 3% in HFKs and 0% in HPV11-transduced HFKs. In regions S2 and S3 relatively few CpGs were methylated in both HPV-immortalized cells and controls. Only in SiHa cells these regions are heavily methylated. More pronounced differences were observed in region S4, which is within the gene body. In HPV18-, 31-, 33-, 45-, 66- and 70-, but not in HPV16-immortalized cells, methylation in S4 was higher than in HFKs and HPV11-HFKs. The 3' part of region S4 was densely methylated in all cells analyzed. No major differences were evident between less oncogenic HPV types (HPV45, 66, and 70), compared to cells immortalized by the more transforming HPV types 16, 18, 31 and 33. However, amongst the different cell lines least methylation was seen in the HPV16-immortalized cells in all four regions.

With respect to the specific transcription factor binding sites previously associated with hTERT regulation in HPV-containing cells, i.e. the X-box motif, E-box motif and CTCF binding sites,^{25, 29-32} minor differences between individual cell lines were observed. The X-box motif in promoter region S1 showed increased methylation in HPV18-, 31-, 33-, 45-, 66- and 70-immortalized cells compared to the controls. However, in HPV16-immortalized cells this site was largely unmethylated. The two E-boxes (c-myc binding sites) were mostly unmethylated, except for a few methylated CpGs in the distal site in HPV18-, and 45-immortalized cells. Both CTCF-sites, located within the gene body in region S2 and S4, were mostly unmethylated, whereas CpGs flanking the CTCF site in S4 were methylated more frequently.

In conclusion, compared to HFK control and HPV11-containing cells, most HPV-immortalized cells showed increased hTERT methylation (with slight differences between the HPV-types). Importantly, the bisulfite sequencing results correspond with the methylation levels determined by qMSP (Figure 1). More methylation was observed in the S3/S4 region where qMSP M2 is located compared to region S1 containing the qMSP M1 region. Moreover, in contrast to qMSP analysis showing significantly increased hTERT M2 methylation at stage 2 in cells that underwent a crisis period, both bisulfite sequencing and qMSP showed no relation to growth behavior at stage 3.

Discussion

The efficiency of keratinocyte immortalization by HPV is HPV-type dependent and may result from differential necessities of supplementary (epi)genetic host cell alterations.⁸ Longitudinal analysis of multiple HPV16-, 18-, 31-, 33-, 45-, 66- and 70-immortalized keratinocyte cell lines revealed a sequential and progressive increase in host gene promoter methylation with passaging. These genes include both (candidate) tumor suppressor genes that are known to become silenced by DNA methylation in the context of hrHPV, such as CADM1, MAL, miR124, PRDM14 and SFRP2,^{15-17, 19, 20} and hTERT, of which methylation has been associated with gene activation.^{18, 21-25}

Methylation of hTERT, miR124-2 and PRDM14 represented the earliest methylation events, methylation levels of which were already significantly increased at the pre-immortal stage (stage 1) compared to controls. Subsequently, at stage 2 a significant increase in ROBO3 methylation was seen. Methylation levels of all four genes progressively increased with passaging. Their rather early onset of methylation is in line with previous results on cervical biopsies, showing methylation in a substantial subset of precursor lesions (CIN3) and increasing frequencies and levels of methylation in cervical carcinomas.^{13, 15, 18, 33}

Methylation of CYGB was identified as a successive event detectable from stage 3 onwards. To the best of our knowledge, this is the first report showing that HPV-mediated transformation is associated with CYGB methylation. In support of this, our preliminary data on cervical biopsies also revealed frequent CYGB methylation in cervical carcinomas and precursor lesions (unpublished results). CYGB methylation has been described in lung-, oral-, breast-, ovarian- and head and neck cancer.^{28, 34-37}

A significant increase in DNA methylation levels of FAM19A4, MAL, PHACTR3 and SFRP2 was seen upon progression from stage 3 to stage 4. CADM1 methylation levels showed a slight increase with passaging, and were particularly high in cancer cell lines. Methylation of these genes has previously also been described in a (major) subset of CIN3 lesions and cervical carcinomas, in line with the cell lines representing a premalignant phenotype.^{13, 16, 17, 38} An earlier onset of MAL methylation compared to CADM1 methylation has been described previously.^{16, 17, 39}

A number of genes remained unmethylated, such as APC, miR124-1, miR124-3 and RASSF1A. Given the fact that these genes are methylated in cervical carcinomas,^{15, 40-44} methylation-mediated silencing of these genes may occur at later stages of transformation and/or may be cell type dependent. In fact, methylation of miR124-1 and miR124-3 was highly frequent in SCC and only occasionally detected in CIN3 lesions.¹⁵ Cell type dependence may particularly account for APC and RASSF1A methylation, which appeared more often in adenocarcinomas than in squamous cell carcinomas.^{14, 40-42, 45} Our *in vitro* models are based on squamous epithelial cells.

Comparison of cells that grew continuously (HPV16-, 18-, 31- and 33-transduced HFKs) and cells that underwent a crisis period (HPV45-, 66- and 70-transduced HFKs), revealed a nearly significant ($p < 0.059$) increase in methylation levels in cells that just escaped from crisis at stage 2. This difference was mainly attributable to significantly increased hTERT methylation. This suggests that methylation of hTERT is particularly advantageous for immortalization of cells harbouring less oncogenic HPV types. Methylation of hTERT has previously been described to result in gene activation and increased hTERT expression is associated with immortalization.^{18, 21-23}

This phenomenon could be confirmed in present study, in which upregulated hTERT mRNA expression, as previously demonstrated in all HPV-immortalized cell lines,⁸ was associated with increased hTERT methylation when compared to hTERT mRNA negative primary cells. However, the CpGs around the transcription start site (region S2) remained largely unmethylated (Figure 3), which was also described by Renaud et al.²⁵ and Jiang et al.⁴⁶ This finding suggests that the core promoter needs to remain unmethylated to allow hTERT activation. Methylation of other regions of the hTERT CpG island has often been associated with elevated hTERT expression.^{18, 21, 22, 25} Two of those regions within the gene contain CTCF binding sites of which methylation is known to hinder CTCF binding.²⁵ It has therefore been suggested that hTERT activation is due to hypermethylation of the CTCF binding sites located at S2 (CpG 2-7) and S4 (CpG 54-56).²⁵ Except for a few CpGs in HPV45- and HPV66-immortalized cells, frequent methylation within the CTCF binding sites was not observed, in contrast to the flanking CpGs, which is in line with our previous findings.¹⁸

It is therefore unlikely that in the studied cell lines hTERT expression is preceded by methylation-mediated inhibition of CTCF binding to established binding sites. Recently, a CTCF-regulated enhancer element 4.5 kb upstream of hTERT, as well as a number of novel candidate CTCF binding sites and non-CTCF repressive elements in the proximal exonic region have been identified, each of which may be affected by DNA methylation.^{47, 48} Moreover, accumulating evidence indicates that the HPV-encoded E6 protein plays a

prominent role in hTERT gene activation by binding to c-Myc or E6AP.^{29, 31, 32, 49} More recently, E6 was shown to inhibit the binding of MAZ, a novel hTERT repressor, which correspondingly increased SP1 binding and gene activation.⁵⁰ The E6 proteins of different HPV-types have different capacities to transactivate hTERT. The E6 proteins of HPV16, 18, 31, 33, 35, 51, 52 and 58 showed highest hTERT promoter activation capacity, whereas E6 of HPV66 and 70 displayed lower activity.⁵¹

In present study least methylation was detected in HPV16-immortalized cells, suggesting that amongst the hrHPV types tested, methylation-mediated hTERT activation may be less essential in case of HPV16E6E7, expression. Except for HPV16, no difference in frequency of hTERT methylation between the strongest (HPV18, 31, and 33) and weakest hTERT activators (HPV66 and 70), as described by Van Doorslaer et al.⁵¹, was seen. Therefore, next to differences in E6 activation capacities, site-dependent methylation patterns or other mechanisms may contribute to hTERT gene activation. Although controversial, HPV16 E6-mediated hTERT activation has previously been suggested to rely (in part) on the proximal E-box.³² Except for methylation of a few CpGs in the distal E-box in HPV18- and 45-immortalized cells, the E-boxes were unmethylated in most immortalized cells.

In conclusion, passaging of HPV16-, 18-, 31-, 33-, 45-, 66- and 70-transduced human keratinocytes is correlated with a progressive increase in DNA methylation of selected host cell genes. The timing of the methylation events differed between genes. hTERT, miR124-2 and PRDM14, were the first genes that became methylated, even prior to immortalization (stage 1). Following immortalization, ROBO3 methylation (stage 2) preceded CYGB methylation (stage 3) followed by CADM1, FAM19A4, MAL, PHACTR3 and SFRP2 methylation. Early onset of selected epigenetic host cell alterations during HPV-induced HFK immortalization was mostly independent on the viral oncogenic capacity. The onset of hTERT methylation was inversely related to the immortalization capacity of the HPV types tested, though diminished with passaging. More detailed analysis of the hTERT regulatory sequences indicated that reactivation of hTERT expression in immortal cells is most likely not due to severe methylation of known repressor sites.

Materials and Methods

Cells and Cell lines

HFKs were isolated from foreskins of independent donors as described before.⁵² Cell cultures containing low-risk HPV11, hrHPV16, 18, 31, 33, 45 and probable hrHPV66 and 70 were established by retroviral transduction of HFKs with the E6E7 open reading frames of the respective HPV-type as described before.⁸ Untransduced HFKs and empty vector (LZRS) transduced HFKs served as controls. HFKs of donor I were transduced with all HPV types. Donor II was transduced with all HPV types except HPV33 and donor III was transduced with HPV16, 33, 45 and 70. Transductants were grown in defined keratinocyte serum-free medium (SFM) (Life Technologies 17005-075) containing 5ng/ml EGF and 50ng/ml bovine pituitary extract, 100 U/mL natrium-penicillin G (Astellas Pharma B.V. 117837/ 315932), 100 µg/mL streptomycin (Life Technologies 11860038), and 2 mmol/L L-glutamine (Life Technologies 25030024) and 80µg/ml geneticin (Sigma-Aldrich G8168) selection. The cervical cancer cell lines SiHa (HPV16) and CaSki (HPV16) and the lung cancer cell line A549 (HPV-negative) were obtained from the American Type Culture Collection (Manassas, VA, USA). Culture conditions were described previously.^{52, 53} All cells were grown at 37°C and 5% CO₂.

DNA isolation and bisulfite modification

Genomic DNA was isolated from cell pellets by proteinase K digestion followed by UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (Life Technology 15593049) extraction as described previously.⁵⁴ Sodium bisulfite modification was performed on 1µg DNA using the EZ DNA Methylation™ Kit (Zymo Research D5002) according to the manufacturer's protocol, which induces chemical conversion of unmethylated cytosines into uracils while leaving methylated cytosines unchanged.

Quantitative methylation specific PCR (qMSP)

DNA methylation of the promoter regions of 14 host cell genes (i.e. APC, CADM1, CYGB, FAM19A4, hTERT, miR124-1, miR124-2, miR124-3, MAL, PHACTR3, PRDM14, RASSF1A, ROBO3 and SFRP2) was determined by qMSP. For hTERT two different regions were analyzed, located in the promoter (M1) and proximal exonic region (M2).¹⁸ miR124-1, -2 and -3 are located at different chromosomal regions and regulated by distinct promoter regions, but all encode the same mature miRNA.¹⁵

QMSPs of single and multiple targets (Table 2) were performed in a 12 µl reaction volume containing 50 ng of bisulfite treated DNA. For single target qMSP QuantiTect Probe PCR Kit (Qiagen 204345) was used and for multiplex qMSP QuantiTect Multiplex PCR Kit (Qiagen 204545).⁵⁵ qMSPs were run on the ABI 7500 and/or ABI 7900 Fast Real-Time PCR System (Applied Biosystems).

Methylation levels were normalized to the housekeeping gene MYOD1 (in case of APC, CYGB and RASSF1A) or ACTB (in case of all other qMSP targets). DNA isolated from cancer cell lines SiHa and A549 served as reference and positive control in the individual qMSPs and were set to 100. SiHa DNA was used as a reference for qMSPs of CADM1, FAM19A4, hTERT, miR124-1, miR124-2, miR124-3, MAL, PHACTR3, PRDM14, ROBO3 and SFRP2 and A549 DNA for APC, CYGB and RASSF1A qMSPs. Methylation levels were determined using the $2^{-\Delta\Delta CT}$ method,⁵⁶ resulting in a quantification of DNA methylation in the HPV-transduced cells relative to the positive controls SiHa or A549. All samples had a ACTB Ct-value <32, indicating sufficient DNA quality.

Bisulfite sequencing

For hTERT methylation analysis by bisulfite sequencing methylation independent PCR (MIP) was performed using 4 primer sets (Table 2) spanning the hTERT promoter and first exon from the coding sequence from -476 to +600 bp relative to the ATG. MIP, cloning and sequencing was performed as described before.¹⁸ Shortly, purified MIP-products were cloned in pGEM-T using pGEM® -T Easy Vector System (Promega A1360) or into the Zero pCR-Blunt II-TOPO vector (Life Technologies K282020). Approximately ten cloned PCR-fragments of every region and cell line were sequenced using the BigDye Terminator v1.1 cycle sequencing kit on an ABI Prism 3100 sequencer (Applied Biosystems). Sequences were analyzed using Chromas Lite version 2.01.

Statistical analysis

Statistical analysis was performed using SPSS (version 20). The average methylation level of all investigated genes as well as methylation levels per gene were compared between stages and between continuously

growing cell lines and cell lineages that encountered a crisis period using the non-parametric Mann-Whitney U test. A two-sided p-value <0.05 was considered statistically significant.

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Conflict of interest

Dr. R.D.M. Steenbergen, Prof. dr. P.J.F. Snijders and Prof. dr. C.J.L.M. Meijer have relationships with Self-screen B.V., The Netherlands.

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Figures

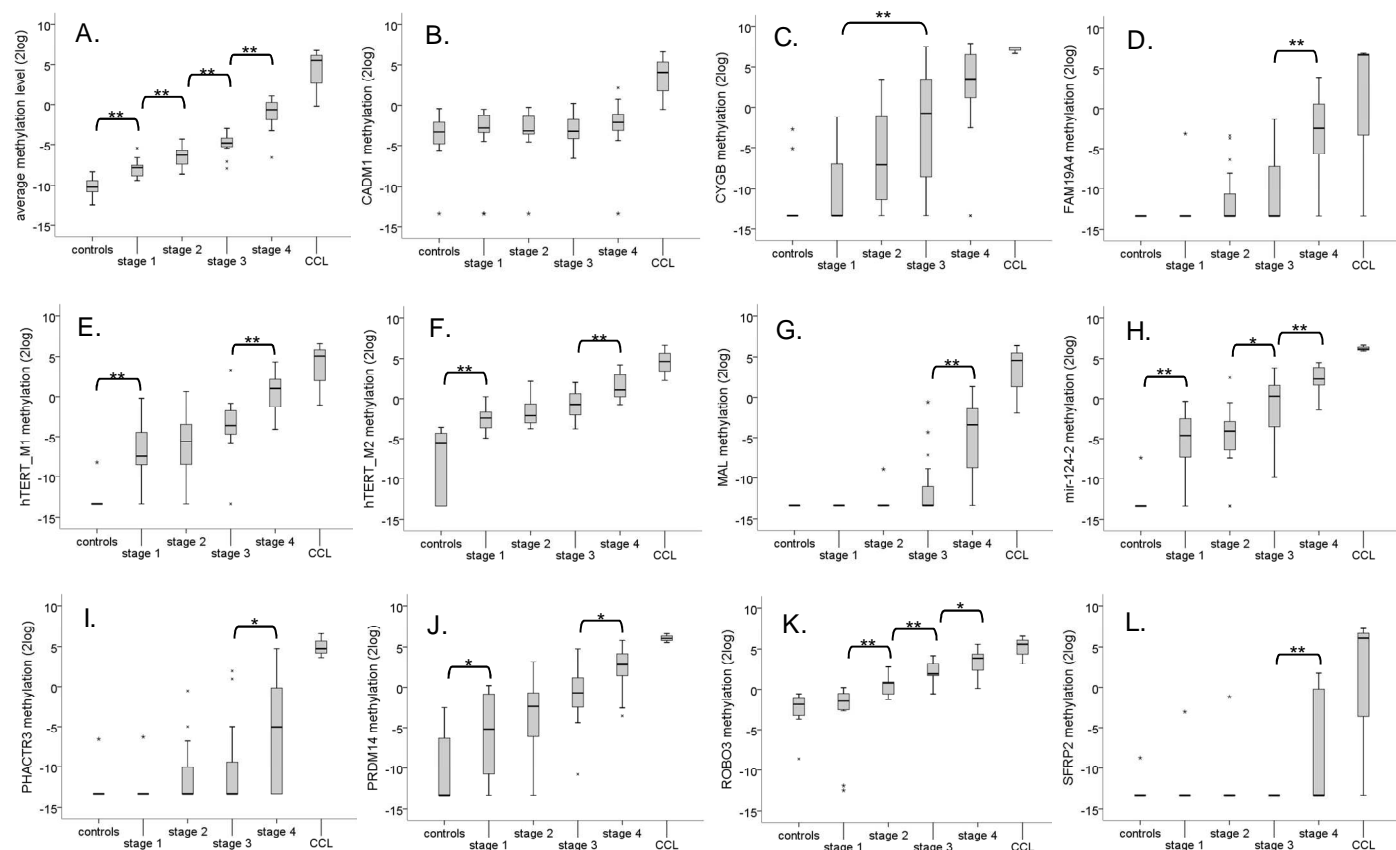


Figure 1: Progressive increase in DNA methylation levels. **A** Box-plot of average methylation level of all 14 genes (APC, CADM1, CYGB, FAM19A4, hTERT, miR124-1, miR124-2, miR124-3, MAL, PHACTR3, PRDM14, RASSF1A, ROBO3 and SFRP2) per stage. **B-L** Boxplots of the methylation levels of the individual gene per stage. Only genes that became methylated in at least one stage are shown (CADM1 (**B**), CYGB (**C**), FAM19A4 (**D**), hTERT M1 (**E**), hTERT M2 (**F**), MAL (**G**), miR124-2 (**H**), PHACTR3 (**I**), PRDM14 (**J**), ROBO3 (**K**) and SFRP2 (**L**)). * p<0,05, ** p<0,01

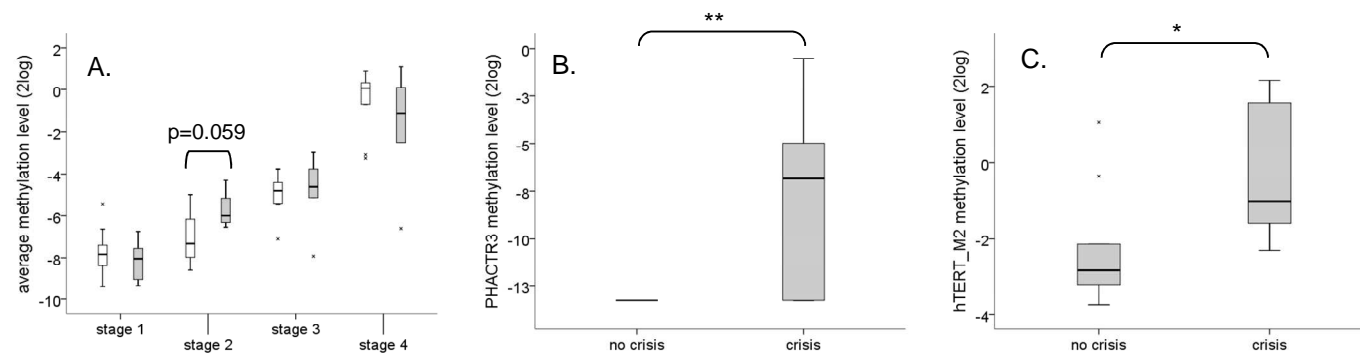


Figure 2: Progressive increase in levels of DNA methylation is mostly growth behaviour independent. **(A)** Boxplot of the average methylation level of all 14 genes (APC, CADM1, CYGB, FAM19A4, hTERT (M1 and M2), MAL, miR124-1, miR124-2, miR124-3, PHACTR3, PRDM14, RASSF1A, ROBO3 and SFRP2) per stage divided between cell lines that grew continuously in culture (white: HPV16, 18, 31 and 33) and cell lines that encounter a crisis period prior immortalization (grey: HPV45, 66 and 70). Significantly differential methylation between cell lines without and with a crisis period prior immortalization was observed for PHACTR3 **(B)** and hTERT M2 **(C)** at stage 2. * $p < 0,05$, ** $p < 0,01$

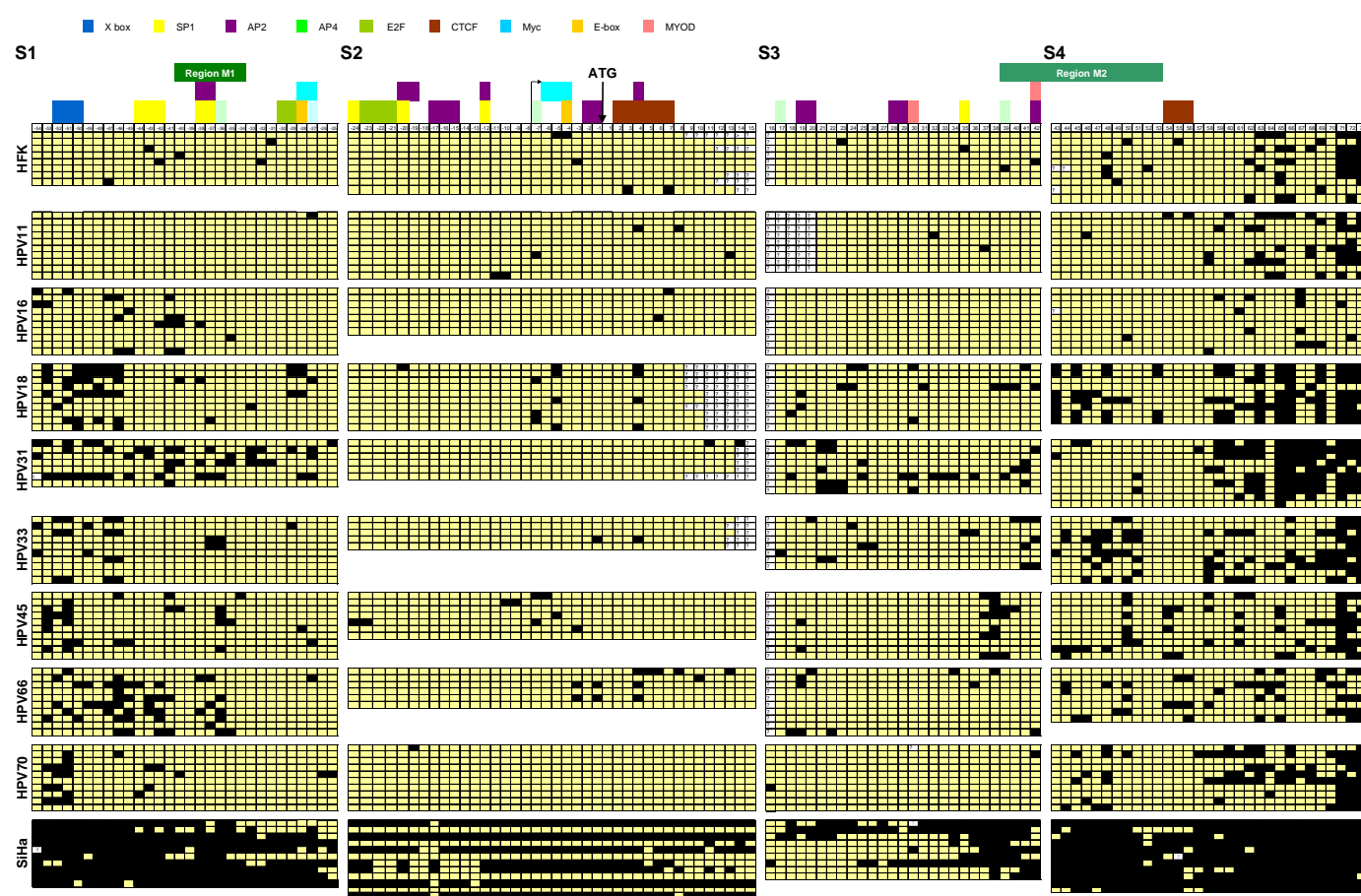


Figure 3: Bisulfite sequencing results of the hTERT CpG island. Overview of the CpG methylation results of individual cloned PCR products of untransduced HFKs, HPV11- and stage 3 HPV16-, 18-, 31-, 33-, 45-, 66- and 70-transduced HFKs of donor I of four regions: S1 spanning -476 to -185 bp, S2 spanning -209 to +96 bp, S3 spanning +90 to +338 bp and S4 spanning +319 to 600 bp relative to the ATG. Numbers refer to the respective CpG and their position relative to the ATG. The position of known and putative transcription factor binding sites are plotted on top of the corresponding CpG. Regions analyzed by qMSP (M1 and M2) are plotted on top of the appropriate CpG in green.

Tables

Table 1: Percentage of hTERT methylation in primary human keratinocytes (HFK), HPV E6/E7 containing HFKs and SiHa cells

	% methylation (total number of clones analyzed)			
	S1 (-476/-185 bp)	S2 (-209/+96 bp)	S3 (+90/+338 bp)	S4 (+319/+600 bp)
Cell lines				
HFK	3 (8)	2 (9)	2 (8)	15 (10)
+HPV11	0 (10)	2 (10)	1 (9)	15 (10)
+HPV16	7 (10)	1 (7)	0 (10)	5 (10)
+HPV18	15 (10)	4 (10)	6 (10)	37 (9)
+HPV31	22 (7)	0 (6)	14 (8)	32 (10)
+HPV33	10 (8)	1 (5)	8 (8)	28 (10)
+HPV45	9 (10)	4 (7)	7 (10)	19 (10)
+HPV66	13 (14)	5 (6)	4 (10)	18 (8)
+HPV70	7 (10)	0 (10)	1 (10)	25 (10)
SiHa	82 (10)	38 (13)	26 (9)	92 (11)

Table 2: Primer and probe sequences

Gene	Forward primer	Reverse primer	probe	length (bp)
Single target qMSP				
hTERT M1	GAGTAG CG TAGG CG ATTTAGGG CG T	GTCCAACAA C CG C GAAC CG AA	CG CACAACCTCTACAACACT CG AACCACCAACTC	75
hTERT M2	TAGATTTT CG GGTT CG TT CG	TCTATACC CG C GAATCCACT	CG ACCTAACCC CG ACA CG CAACTA	132
MAL	TTAGGTTATTGGGTTT CG C G	GTACTAA CG T CG ACCTTAA AC CG A	T CC CG C G CAAACCTCT CG CTAAC	86
miR124-1	CG G C G GGGAGGATGTT	ATAAAAA CG A C G C G TATAC GT ACG	CG G C G TTTTTTATTTTT	94
mir124-3	AC G C G G C G AAGAC CG TTT	CG A AC CG A C G A AC CG T CG AAA	AAAATCCT CG CCC G AAAA AC CG C G A	95
ROBO3	AGGAGGAGGGTAC CG AAGAGGTATC	AAACCC CG TAAACTAA AC CG TAAAC	CC GC TCTCCTAC CG ATAC GC CTAAATAC CG AT	120
ACTB	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATA AA ACCTACTCCTCCCTTAA	ACCACCACCCAACACACAATAACAAACACA	
Multiplex qMSP				
APC	GAACCAAA AC G CTCCCAT	TTATATGTCGGTTACGTGCGTTTATAT	CCC GT CG AAAACC CG CC CG ATTA	74
CADM1	CG TATGTTATTAGTATTTTATTAGTTGTT CG TT C	CG CT CG ACAACACTACACT CG	ACCTACCTCAA ACT A AC CG AC GT TA ACTACCT CC G A	106
CYGB	CG AGGT CG AT CG TTAGTT CG TT C	CCAAC CG ACTAACT CG AAAA CG C G	CG G C G GT CG T CG TGGATT T AG	117
FAM19A4	AGT CG GG CG GTT CG GTTA	CCAAA CG A C G C G CAACTA	CCCAACTA AC CG C G CTAA	106
miR124-2	GGGTAATTAATTTGGATTTAC GT CG TTAT	CG TAAAAATATA AA CG ATAC GT ATACCTAC GT	TTTACAACACAC CG CCTAAA	138
MYOD1	CCAAC T CCAATCCCTCTCTAT	TGATTAATTTAGATTGGGTTTAGAGAAGGA	TCCCTTCCTATTCTAAATCCAACCTAAATACCTCC	162
PHACTR3	GGTTATTTT CG A G C G GTTT C	CG AATACTCTAATTCCAC CG C G ACT	AAC CG C GT CG AAAA AC G AAAA CG ACTAC	114
PRDM14	TTAC GT GTTATTGT CG GGGATT C	ATATCTATTCCTAATACCTAAAA AC G AA AC G	AAAC GC CCTTAA AC GT AAAA ACT T CG CCTC	88
RASSF1A	GC GTGAAGT CG GGGTT C	CCC GT ACTT CG CTAACTTTAA AC G	ACAA AC CG C G AA CC G AA AC G AAACCA	75
SFRP2	GAGTAG CG TAGG CG ATTTAGGG CG T	TCC CG AA CC CG CTCTCTT	CG CTAAATAC CG ACT CG AAACCC CG AA	69
Bisulfite sequencing				
hTERT S1	GTTTTTAGGGTTTTTATATTATGG	AAACTAAAAATAAAAAACAAAAC		292
hTERT S2	GTTTTGTTTTTTATTTTTAGTTT	AACCCTAAAACCCCAA		305
hTERT S3	TTGGGGTTTAGGGTTG	ACCAACTCCTTCAACAAAA		248
hTERT S4	GTAGGTGTTTTGTTGAAGGA	AACTAAAAACCAACACAA		281

CpGs are indicated in bold